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Quantum dot induced acute changes in lung mechanics are mouse strain dependent

David K. Scovillea, Collin C. Whitea, Dianne Bottaa, Dowon Anb, Zahra Afsharinejad, Theo K. Bammlera, Xiaohu Gaoc, William A. Altemeierb and Terrance J. Kavanagha

aDepartment of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, USA; bDepartment of Medicine, University of Washington, Seattle, WA, USA; cDepartment of Bioengineering, University of Washington, Seattle, WA, USA

ABSTRACT

Introduction: Concerns have been raised regarding occupational exposure to engineered nanomaterials (ENMs). Potential impacts on lung function from inhalation exposures are of concern as the lung is a sensitive ENM target in animals. Epidemiological data suggest that occupational exposure to ENMs may impact respiratory and cardiovascular health. Quantum dots (QDs) are ENMs with outstanding semiconductor and fluorescent properties with uses in biomedicine and electronics. QDs are known to induce inflammation and cytotoxicity in rodents and high dose exposures impact lung function 2 weeks after exposure. However, effects of mouse strain and the temporality of QD effects on lung function at more occupationally relevant doses have not been well-established.

Objective: We evaluated the impact of QD exposure on respiratory mechanics in C57BL/6J and A/J mice. Previous work found a greater initial inflammatory response to QD exposure in A/J mice compared to C57BL/6J mice. Thus, we hypothesized that A/J mice would be more sensitive to QD-induced effects on lung mechanics.

Methods: C57BL/6J and A/J mice were exposed to 6 µg/kg Cd equivalents of amphiphilic polymer-coated Cd/Se core, ZnS shell QDs via oropharyngeal aspiration. Lung mechanics were measured using forced oscillation, and inflammation was characterized by neutrophils and cytokines in bronchoalveolar lavage fluid.

Results: Both strains showed signs of QD-induced acute lung inflammation. However, lung mechanics were impacted by QD exposure in A/J mice only.

Conclusions: Our findings suggest that susceptibility to QDs and similar ENM-induced changes in lung function may depend at least in part on genetic background.
generate aerosols), or from accidental releases into the working environment.

In vivo studies in rodents have shown that pulmonary exposure to QDs can cause both acute and chronic inflammation characterized by neutrophil influx, cytokine production, compromised alveolar/capillary barrier function and granuloma formation (Jacobsen et al., 2009; Ma-Hock et al., 2012; McConnachie et al., 2013; Roberts et al., 2013; Scoville et al., 2015). In some cases, cytotoxicity and DNA damage were also observed (Jacobsen et al., 2009; Roberts et al., 2013). Changes in coatings have been shown to modify the toxic and inflammatory potential of QDs (Ma-Hock et al., 2013; Roberts et al., 2013). Furthermore, QDs have been shown to cause changes in lung mechanics when measured 17 days after intratracheal installation of a high dose of QDs (Ho et al., 2013). However, the ability for QDs to impact lung mechanics in the acute setting has not been evaluated. The role of mouse strain has also not been evaluated for ENM effects on lung mechanics. The role of mouse strain has also influenced by mouse strain. We used forced oscillation techniques to assess lung mechanics when measured 17 days after intratracheal installation of a high dose of QDs (Ho et al., 2013). However, the ability for QDs to impact lung mechanics in the acute setting has not been evaluated. The role of mouse strain has also not been evaluated for ENM effects on lung mechanics.

In this study, we sought to determine whether pulmonary exposure to QDs elicits changes in mouse lung mechanics in the acute setting and, if so, whether such effects would be influenced by mouse strain. We used forced oscillation techniques, thought to currently be the most accurate method for measuring mouse lung mechanics (Irvin and Bates, 2003; Vanoirbeek et al., 2010). Since there is some concern over occupational health effects of ENM exposure, such as aggravation of asthma, and some ENMs have been shown to enhance models of allergic airway disease in mice, we used a methacholine challenge to assess airway hyperresponsiveness (AHR) (Brandenberger et al., 2013; Golan et al., 2012; Hargreave et al., 1981; Jonasson et al., 2013; Nygaard et al., 2009; Postma and Kerstjens, 1998; Roy et al., 2013).

We have previously observed that C57BL6/J were more resistant than A/J mice to acute QD-induced lung inflammation at occupationally relevant doses (Scoville et al., 2015), and thus used these mouse strains to test the hypotheses that (i) QDs negatively impact mouse mechanics and (ii) that responses are more pronounced in A/J mice. Results from this study showed that QDs induced lung inflammation in both C57L/J and A/J mice and that A/J mice were more susceptible to the impact of QDs on lung mechanics. These mouse strain differences underscore the importance of conducting such studies in mouse strains known to exhibit differences in airway reactivity, and also suggest that the potential for similar ENMs to affect lung function in humans could be influenced by an individual’s genetics.

Material and methods

Ethics statement

All procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC) on protocol #2384-08.

Animal housing and dosing

C57BL6/J and A/J mice (n = 9 for QD and saline groups for both strains) were ordered from Jackson Laboratories (Bar Harbor, ME) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved vivarium under modified specific pathogen-free (SPF) conditions at the University of Washington. Food and water were available ad libitum during the 1-week acclimation period. Mice were dosed via OPA with either 6 μg Cd equivalents/kg body weight of a 10 nM TOPO-PMAT QD solution or an equivalent volume of sterile saline as previously described (Scoville et al., 2015).

Pulmonary function testing

Twenty four hours after dosing, QD or saline-treated C57BL6/J or A/J mice (N = 6 for each category) were anesthetized with pentobarbital (90 mg/kg body weight), intubated via tracheotomy with an 18 gauge Luer stub that was secured in the trachea with a suture. The mice were then connected to a flexiVent 5.2 instrument (SCIREQ USA Inc., Tempe, AZ). After an injection of pancuronium (0.1 mL) to eliminate reflexes from the mouse for breathing, pulmonary function was evaluated. The lungs were ventilated with a tidal volume of 10 mL/kg, at a rate of 180 breaths/min, with a positive end expiratory pressure of 3 cm H2O. Subsequently, a total lung capacity maneuver was performed at 30 cm H2O and baseline measurements were collected. To assess AHR, progressive doses of nebulized methacholine (MTCH) (0, 3.125, 12.5 and 50 mg/mL) were delivered to the lungs over 15 s periods. At each dose of MTCH, a 3 s multi-frequency forced oscillation maneuver was applied for determination of total lung impedence followed by a 1.5 s single-frequency oscillation for determination of Resistance (R), Elastance (E) and Compliance (C). Airway Resistance (Rn), Tissue Damping (G) and Tissue Elastance (H) were calculated by the flexiVent software by applying the Constant Phase Model to the impedance measurement. Hysteresivity (eta) is the ratio of G/H. Area under the curve (AUC) was calculated for individual mice for comparisons between strains and treatment groups (Vanoirbeek et al., 2010).

Bronchoalveolar lavage

After pulmonary function testing, the animals were euthanized with an overdose of pentobarbital and bronchoalveolar lavage (BAL) was performed. Mice that did not undergo lung mechanics testing were euthanized using CO2 narcosis followed by cervical dislocation (N = 3 for each category). Three 1 mL lavages were performed on each mouse using phosphate-buffered saline (PBS). BALF was centrifuged at 500g for 10 min and cells from the three lavages were pooled for flow cytometry. Supernatant from the first lavage was frozen at –80°C for measuring total protein and cytokines.

BALF protein and cytokine analysis

BALF total protein was measured using the Bio-Rad Protein Assay (BioRad, Hercules, CA). Protein concentrations were interpolated from a standard curve created using dilutions of a BSA solution (0–0.35 mg/mL). BALF cytokines IFN-γ,
IL-5, IL-17A, IL-33, KC (CXCL1) and IL-10 were measured in duplicate using a U-Plex assay (Meso Scale Discovery; Meso Scale Diagnostics, Rockville, MD). Samples below the lower limit of detection (LLOD) were replaced with the analyte-specific LLOD divided by the square root of 2.

**Lung tissue and glutathione**

Following BAL, the right lung was harvested and flash frozen in liquid nitrogen for determination of glutathione concentration, and the left lung was fixed in 10% neutral buffered formalin (NBF) at 20 cm H2O. Levels of total glutathione were measured as previously described (Weldy et al., 2011). Briefly, glutathione in lung tissue samples and standards (0–0.25 mM) was reduced with TCEP [tris(2-carboxyethyl) phosphine], and then derivatized with naphthylene-2,3-dicarboxaldehyde. Relative fluorescence intensity was measured for samples and GSH standards. Sample concentrations were interpolated from the GSH standard curve. Standards and samples were analyzed in triplicate and samples were normalized to total protein levels in the lung tissue sample measured using the Bradford method.

**Flow cytometry**

Combined BALF cells were prepared and analyzed using flow cytometry as previously described (Scoville et al., 2015; Weldy et al., 2011). Briefly, cells were stained with an antibody cocktail containing a phycocerythrin-conjugated primary antibody against Cd11b (AbCam, Cambridge, MA), a biotinylated primary antibody/streptavidin/AlexaFluor350 secondary antibody combination against Ly-6G/Ly-6C (Gr1) (BioLegend, San Diego, CA), and an Alexafluor 488 conjugated primary antibody against F4/80 (eBioscience, San Diego, CA). Cells high in Cd11b and low in F4/80 fluorescence were classified as neutrophils.

**Statistical analysis**

The data collected in this study were analyzed using Graphpad Prism (GraphPad Software, La Jolla, CA) and R (R Core Team 2014). All data were log transformed prior to statistical analysis. Area under the methacholine dose–response curve was calculated for each animal, and a two-way analysis of variance (ANOVA) was used to assess interactions between mouse strain and QD treatment effects. When no statistically significant interaction was observed between QD treatment effects and mouse strain, within strain t-tests were performed to compare saline and QD-treated mice when significant ANOVA treatment effects were observed. Two-way ANOVA was also used to determine if there were baseline differences in measures between mice that underwent lung mechanics testing and those that did not. We included all mice in our analysis to evaluate baseline differences. Concentrations of total BALF protein. To allow for comparisons across measurements, we only further evaluated levels of total protein from mice that underwent lung mechanics testing. Cytokines were assessed in mice that underwent lung mechanics testing and that represented high, moderate and low % neutrophils in BALF (N = 3 for each category). Pearson’s correlation coefficients between endpoints were calculated using r.corr function in the Hmisc R package (Harrell & Dupont, 2016). Data sets were managed in Microsoft Excel and R. Plots were generated using ggplot2 (Wickham 2009) and corrplot (Wei & Simko 2016).

**Results**

**Lung mechanics**

Similar to a study by Nolin et al. (2016), we used total lung resistance (R) as our measure of AHR. We observed that QD treatment and mouse strain significantly affected AHR overall (Figure 1(A)). Total lung elastance (E) was significantly affected by QD treatment in A/J but not C57BL/6J mice (Figure 1(B)). Total lung compliance (C) was significantly affected by mouse strain but not QD treatment (Figure 1(C)). Airway resistance (Rn) was also significantly affected by mouse strain (Figure 1(D)). Tissue damping (G) was significantly increased by QD treatment in A/J mice but not in C57BL/6J mice (Figure 1(E)). Hysteresivity (eta), which is the ratio of G/tissue elastance (H), was significantly affected by mouse strain overall but not by QD treatment (Figure 1(F)). Supplementary Table S1 shows the parameters of two-factor ANOVA analyses.

**BALF neutrophils, total protein and cytokines**

We observed that % neutrophils in BALF was significantly increased in QD-treated C57BL/6J and A/J mice compared to saline-treated strain-matched controls (Figure 2(A)). In order to characterize potential drivers of the acute QD inflammatory response, we measured cytokines representative of Th1 (IFN-γ) and Th2 (IL-5, IL-33) immune responses as C57BL/6J mice are thought to be more Th1 response prone and A/J mice are thought to be more Th2 prone (Hallstrand et al., 2013; Schroder et al., 2004; Sellers et al., 2011). We also measured IL-17, which has been associated with neutrophil recruitment (Choy et al., 2015). Levels of total protein and IL-33 in BALF were not different between the two mouse strains or between treatment groups (Figure 2(B,C)). Levels of the neutrophil chemokine KC were significantly increased in QD-treated C57BL/6J mice compared to saline (Figure 2(D)). We also found a significant association between levels of KC and BALF neutrophils (Figure 2(E)). IL-10, an anti-inflammatory cytokine, is often increased in concert with pro-inflammatory cytokines in order to limit tissue injury associated with inflammation (Saraiva & O’Garra 2010). However, IL-10, as well as IL-17, IFN-γ and IL-5 were undetectable in virtually all mice (data not shown). Supplementary Table S1 shows the parameters of two-factor ANOVA analyses.
Lung glutathione

Levels of lung glutathione were significantly different between the two mouse strains overall (Figure 3(A)). Additionally, we observed significant inverse correlations between total lung glutathione levels and the lung mechanics measures R and G in QD-treated mice (Figure 3(B,C)). Supplementary Table S1 shows the parameters of two-factor ANOVA analyses.
Discussion

In this study, we investigated the impact of mouse strain and QD treatment on lung mechanics. The results indicate that QDs can influence mouse lung mechanics in a strain-dependent manner (Scoville et al., 2015). We observed that QD treatment significantly increased tissue damping (G) and lung elastance (E) in A/J mice but not in C57BL/6J mice. We also found that AHR was significantly affected by QD treatment and mouse strain. Other measures of lung mechanics [airway resistance (Rn), compliance (C) and hysteresivity (eta)] were significantly affected by mouse strain, but not by QD treatment.

The results from measuring lung mechanics indicate that G and E are sensitive to QD treatment. Lung elastance (E) captures the elastic recoil properties of the lung. Increases in E were previously observed with silica nanoparticle instillation (Ferreira et al., 2013). Tissue damping (G), referred to as Gti by Tomioka et al. (2002) captures the dissipation of air pressure wave energy into the lung tissues. True parenchymal tissue resistance to airflow (Rti) has been measured directly using alveolar capsules, and although G and Rti are not directly comparable, both parameters were significantly affected in a mouse ovalbumin (OVA) allergic airway model (Tomioka et al., 2002). Furthermore, G was found to be a more sensitive measure than Rn (referred to as Raw by Tomioka et al., 2002), highlighting the importance of the peripheral lung tissue to overall lung response. In a review article referencing the same OVA study by Tomioka et al. (2001) it was suggested that changes in peripheral lung tissue and small airways may be related to changes in lung secretions (Irvin & Bates, 2003; Tomioka et al., 2002). The effects of lipopolysaccharide (LPS) on mouse lung mechanics and pulmonary surfactant have also been studied (Ingenito et al., 2001). Surfactant proteins are an essential component of the alveolar lining fluid, where they act to reduce surface tension. LPS was found to alter lung mechanics and change the expression and function of surfactant proteins (Ingenito et al., 2001). The authors suggested that inflammation in the lungs could damage surfactant proteins and lipids through neutrophil-derived proteases, reactive oxygen species and lipid inflammatory mediators (Ingenito et al., 2001). In the context of these other studies that investigated mouse lung mechanics and inflammation, we interpret our findings as evidence that acute exposure to QDs can adversely impact peripheral lung tissue mechanics and that A/J mice are more sensitive compared to C57BL/6J mice. A study comparing Aspergillus fumigatus-induced allergic inflammation and AHR between C57BL/6J and BALB/c mice attributed resistance in C57BL/6J mice to increased levels of surfactant protein D (Atchochina et al., 2003). Interestingly, a study investigating MWCNT effects on lung mechanics in a non-allergic model found significant MWCNT-associated increases in G in C57BL/6J mice (Wang et al., 2011) suggesting that susceptibility to ENM-induced changes in lung mechanics are both particle and strain specific.

The observed inverse correlations of R and G with lung glutathione in QD-treated mice in our study are consistent with previous reports indicating that glutathione supplementation improved LPS induced changes in lung mechanics and inflammation and that glutathione mediates lung permeability associated with cigarette smoke and air pollution PM10 (Rahman & MacNee, 1999; Sharma et al., 2011). Furthermore, in a previous study (Scoville et al., 2015), we also observed an inverse correlation between lung glutathione content and inflammatory responses to QDs among eight genetically extant mouse strains (the Collaborative Cross parental strains which include C57BL/6J and A/J). Together, the findings from the present investigation and from our previous study (Scoville et al., 2015) lend support to the suggestion that QD effects on lung inflammation and mechanics are at least in part related to their ability to cause oxidative stress.

We also observed a QD-induced inflammatory response characterized by significantly increased % neutrophils in BALF in both C57BL/6J and A/J mice and levels of KC in C57BL/6J mice. This is interesting in the context of our previous study showing that C57BL/6J did not exhibit QD associated significant increases in KC or in % neutrophils in BALF 8 h after QD exposure (Scoville et al., 2015). In contrast, A/J mice showed significant increases in KC 8 h after QD exposure, and, in a sensitivity analysis where potential outliers were removed, significant increases in BALF neutrophils (Scoville et al., 2015). These results suggest kinetics of QD-induced inflammatory response are also mouse strain
dependent. The inability to detect IL-5, IFN-γ or IL-17 suggests that T-helper cells were not major contributors in the acute QD-induced immune response. Furthermore, absence of detectable IL-10 possibly indicates that this response has not yet transitioned to the resolution phase by 24h. The relationship between % neutrophils and BALF KC in this study was comparable to that observed previously at 8h across all eight Collaborative Cross founder strain mice (Scoville et al., 2015).

In conclusion, the results from this study suggest that QD effects on lung mechanics are mouse strain dependent (with A/J mice being more affected than C57BL/6 mice), and that peripheral lung tissue may be more sensitive to QDs than central airways. The observation of mouse strain differences in QD-induced changes in lung mechanics suggest that the potential for ENMs to affect lung function in humans could depend on genetics. Observations of QD-induced lung inflammation in this study and in previous studies could have implications for predisposition to infection and other conditions. The results from this study showing that QDs enhanced AHR in general warrant future studies to investigate the impact of QDs on allergen-sensitized mice. Inverse associations between QD-induced AHR and glutathione warrant future studies to further investigate the biochemical and pathophysiological basis for this relationship.

Disclosure statement
No potential conflict of interest was reported by the authors.

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